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13. ABSTRACT *(Maximum 200 Words)*

We wished to ascertain whether the failure to inherit putative protective alleles of HLA class II genes is linked to the development of breast cancer. We molecularly typed HLA DPB1, DQB1, DRB1 and DRB3 alleles in 176 Caucasian women diagnosed with early onset breast cancer, and in 215 ethnically matched controls. HLA DQB*03032 was identified in 7% of controls but in no patients with early onset breast cancer ($p = 0.0001$). HLA DRB1*11 alleles were also significantly overrepresented ($p < 0.0001$) in controls (16.3%) as compared with patients with early onset breast cancer (3.5%). HLA DQB*03032 and HLA DRB1*11 alleles may have a protective role in human breast cancer.

We have identified a protein expressed at very low levels during development and which is expressed at very high levels in a proportion of breast tumors, and may therefore represent a relevant antigen. This protein, a kinase called PKK, was cloned in our laboratory and has been shown to functionally connected to a kinase downstream of the EGF receptor, suggesting that high level PKK expression may be relevant to breast tumorigenesis. In unpublished studies by another group, this protein was identified by screening expression libraries using sera from breast cancer patients.

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INTRODUCTION

While it is likely that genetic susceptibility plays a role in the development of most human cancers, evidence supporting this view has hitherto been obtained in only a small fraction of patients who typically carry germline mutations in tumor suppressor genes. In addition to the widely recognized role of acquired alterations in oncogenes and tumor suppressor genes, considerable evidence exists to suggest that the immune system might play a protective role in tumorigenesis. Although immune surveillance is believed to be involved in the elimination of tumors, immunotherapeutic approaches to human cancer have by and large proved unsuccessful. In this study we have used molecular approaches to type all HLA class II DP, DQ, and DR alleles in patients who were diagnosed with breast cancer before the age of 40, and in ethnically matched controls. The goal was to identify if any specific MHC class II allele is linked either positively or negatively in this disease.

BODY

We reasoned that performing a detailed molecular analysis of HLA DPB, DQB, and DRB alleles in patients with early onset breast cancer and ethnically matched controls might provide information as to the potential existence of alleles that could confer susceptibility or resistance to this human cancer. **Very few studies of sufficient power (in term of numbers of subjects and numbers of alleles) have been performed that address the issue of HLA class II polymorphisms for any non-viral human tumor. No studies on the molecular typing of HLA class II alleles in breast cancer had been reported prior to our study (see Appendix; reference 1).** We performed detailed molecular typing of HLA class II alleles in 217 Caucasian controls and 179 Caucasian women with early onset breast cancer (diagnosis at or before age 40). We chose to study **early onset breast cancer** because it is known that genetic predisposition is more likely to influence tumor progression in this patient subset (13). **Two extremely strong negative associations with early onset breast cancer were noted, as described below, one with DQB*03032 ($p = 0.0001$), and the other with DRB1*11 ($p < 0.0001$).**

Technical details of the typing methods used are summarized in the Appendix and will not be described here in detail. The PCR SSO method was used for typing (in conjunction with the sequencing of PCR products when an allele identification was in doubt). No strong negative or positive associations were noted for any DPB1 alleles in patients with breast cancer. Modified Bonferroni corrections were made (for the number of comparisons) according to the method of Svejgaard and Ryder (2).

Ethnicity

A major concern in any population-based study is the role of ethnicity. The vast majority of the patients and controls that were available for study were Caucasians. A few individuals in each of the original study groups were of Asian, Hispanic, or African-American origin and were excluded from the analysis presented above (because the numbers accumulated to date will not permit meaningful

statistical analysis). The Caucasian patient group contained 17 Jewish subjects, and 13 Jewish controls were studied. Data was analyzed for all Caucasians and also separately for non-Jewish and Jewish subjects.

*DQB*03032 is a putative resistance allele for early onset breast cancer*

14 out of 199 controls and none of 176 patients with breast cancer inherited the DQB*03032 allele (p=0.0001). The Relative Risk was 0.0358. The corrected p value (corrected for the number of comparisons made) is 0.0016, which remains highly significant. When the data was separately analyzed excluding Jewish patients and controls, the negative association of DQB*03032 in breast cancer remained highly significant (p=0.0002). The numbers of Jewish patients and controls was insufficient for this sub-group to be analyzed separately in a statistically meaningful manner. None of the Jewish patients (n=17) or controls (n=13) inherited DQB*03032.

*DRB1*11 alleles are also negatively associated with breast cancer*

The only allele or group of alleles at the DRB1 locus which was inherited in a significantly different fashion between patients and controls was DRB*11, which was typed in 35 controls but in only 6 patients with breast cancer (p<0.0001). The Relative Risk was 0.1846. The corrected p value for this negative association is <0.0030, which is also highly significant. When Jewish subjects were excluded from analysis the negative association of DRB1*11 in non-Jewish Caucasians remained highly significant (p=0.0006). Although the numbers of Jewish subjects was small, it was nonetheless intriguing that 5 out of 13 Jewish controls (38.5%) and 0 out of 17 Jewish patients (0%) inherited DRB1*11 (p= 0.0090). Clearly a large study needs to be undertaken involving Jewish women with breast cancer and ethnically matched controls.

Our studies suggest that DQB* 03032 and DRB1*11 may represent resistance alleles for breast cancer. It is theoretically possible that DQB*03032 is in linkage disequilibrium with an unidentified growth regulating gene, a polymorphic allele of which dominantly suppresses mammary tumorigenesis. If such a polymorphic linked dominant tumor suppressor gene exists, an allele of this gene would be expected to form an extended haplotype in conjunction with DQB*03032, and a specific linked DRB1* allele. The failure to note a negative association in breast cancer with any one of the three DRB1 alleles known to be in linkage disequilibrium with DQB*03032 in Caucasians argues against the theoretical possibility that DQB*03032 is in linkage disequilibrium with an unknown dominant tumor suppressor gene. However, the existence of such genes cannot be ruled out. If additional and distinct negative associations with breast cancer are found in future studies on other defined ethnic populations, the existence of unknown polymorphic dominant non-HLA tumor suppressor genes in linkage disequilibrium with the HLA locus may be considered even less likely.

We have pursued two directions since we published the findings outlined above. Our goals are to:

1. verify that protective HLA class II alleles can be confirmed in a separate cohort of early onset breast cancer patients

2. identify potential antigens in breast tumors which could potentially provide peptides that associate with HLA class II molecules

There was an untested but theoretically valid reason for our originally choosing to focus on patients with early onset breast cancer. We reasoned that genetic differences were more likely to show up in such a population. We had not originally expected that differences in the inheritance of HLA class II alleles would be as striking as they were when we completed our analyses on Caucasian early onset breast cancer patients and controls. The virtual absence of specific HLA class II alleles in women with early onset breast cancer was unexpected. Our data very strongly suggested that protective HLA class II alleles might exist for breast cancer. It appeared very likely that we had been able to discover these potentially protective alleles precisely because we had studied patients with early onset breast cancer, rather than older patients with a later onset of disease. At this point we recognized that the potential existed to take this work to its logical conclusion which would be to:

1. confirm that protective HLA class II alleles exist in a second ethnic population, and if this was confirmed,
2. define peptides that might be presented by these alleles, and
3. use these two pieces of information to develop a novel therapeutic vaccination strategy, i.e. eventual reintroduction of protective HLA class II alleles in women with early onset or sporadic breast cancer, in conjunction with a specific protective peptide.

According to our Statement of Work we had originally proposed to follow up our studies on early onset breast cancer by examining HLA class II alleles in patients with sporadic breast cancer. The main reason for including patients with sporadic breast cancer was that they were easily available and we knew that there was only a finite and limited number of patients with early onset breast cancer available locally through three major Boston area cancer centers / hospitals. In our previously submitted report we had stated that "Although sporadic breast cancer patients are readily available for study at the MGH Cancer Center, we have opted to keep our focus on early onset breast cancer, since associations with HLA class II alleles may show up more readily in this group".

Indeed, samples from a very large number of sporadic breast cancer patients are already available in the MGH Cancer Center. Although it would have been relatively easy to focus on those patients in the latter portion of this project, our very dramatic results suggested to us that it was critical to obtain more samples from patients with early onset breast cancer. We very strongly felt that we might have been successful in identifying putative protective alleles precisely because we had chosen to study women who develop cancer early, because genetic factors are more likely to be readily revealed in such a population. It is possible that in older women with sporadic breast cancer multiple epigenetic events potentially contribute to the failure of immune surveillance. Protective HLA class II alleles, while relevant, might not be readily revealed in such populations. We therefore felt

that while there is certainly scientific value in also studying older subjects with breast cancer, the greatest priority needed to be given to verifying that protective alleles for breast cancer actually exist. Clearly, going by previous experience in the field with other associations between HLA class II alleles and disease, verification would best be obtained by looking at ethnically matched sets of early onset breast cancer patients and controls from additional and distinct defined ethnic backgrounds.

We therefore continued analyzing early onset breast cancer patients and controls of Caucasian and Ashkenazi Jewish origin obtained from Massachusetts General Hospital, Brigham and Women's Hospital, and the Dana Farber Cancer Institute. We have since included every available sample in our ongoing studies. However, as the number of non-Caucasian early onset breast cancer patients available for study in Boston is limited we established a collaboration with Dr. O.I. Olopade at the University of Chicago who kindly made available 185 African-American early onset breast cancer patient samples as well as ethnically matched controls, and also samples from a cohort of ethnically matched Nigerian early onset breast cancer patients and controls (approximately 200 patients and 180 controls). We therefore embarked on a major set of studies seeking to confirm (or contradict) our observations by examining a second Caucasian population, a more complete and statistically valid Ashkenazi Jewish population, an African-American population, and a Nigerian cohort. We also separately established a collaboration with Dr. Bruce Ponder in Cambridge, England, to ensure that we have statistically significant numbers of Caucasian and Ashkenazi Jewish early onset breast cancer patients and controls. Given the obvious potential therapeutic importance of protective HLA class II alleles we believe that a large study, at the very least involving a second ethnic group, is essential to establish that protective alleles in early onset breast cancer represent a solid target for future therapy. We therefore did not choose to avoid studying more patients with sporadic breast cancer, but actually extended our studies in the final stage of our studies, continuing to focus on early onset breast cancer patients. In the original Statement of Work, we had chosen to continue studies on patients that we knew were readily available locally. Based on our results we did choose to continue to study patients with breast cancer but to retain our focus on the more difficult to obtain group of early onset breast cancer patients. While this does represent a slight deviation from the original Statement of Work, it actually represents scientifically relevant studies very similar to those proposed, which were initiated 14 months ago, and are still in progress. I apologize for not clarifying this sharpening of our focus earlier. When these studies are completed and published we certainly plan to acknowledge the support from our USAMRMC grant, although clearly we have had to subsequently seek funds from other agencies to continue these studies. (We are partly funded at present by a grant from the Avon Foundation and have applied to the National Institutes of Health for further support. This has now been included under Reportable Outcomes in the revised report).

Ideally we would like to identify a tumor specific antigen in breast tumors which may yield peptides that are presented by protective HLA class II alleles. Identification of a specific HLA class II allele and a specific protective peptide may facilitate the development in the future of possible gene therapy approaches to the treatment of breast cancer.

What is a tumor specific antigen? Proteins that are mutated in tumor cells may constitute tumor antigens, or proteins that are highly overexpressed in tumors but which are expressed at low levels during early development may also function as tumor antigens. Intriguingly some tumor antigens are detected by the fact that patients with cancer make antibodies against these proteins. These antibodies do not have protective value. They however help identify mutated or amplified genes in many tumors. Patients with hematologic malignancies commonly harbor antibodies directed against (mutant) p53. Antibodies against telomerase and WT1 (both of which are overexpressed as "wildtype" proteins in ceratin malignancies) are also frequently seen in patients with leukemias.

We have analyzed available microarray data to identify genes that are expressed at high levels in breast cancer. Eventually an appreciation of the "anchor residues" for specific HLA class II alleles will help us narrow down the number of candidate proteins. In separate studies we cloned a novel kinase which we call PKK which is expressed in a highly restricted manner during early murine development (3). The catalytic loop region of PKK contains a Ser-X-X-X-Ser motif typically found in MAPkinase kinases (MAP2Ks). While searching for a MAPkinase kinase kinase (MAP3K) that might phosphorylate and activate PKK we discovered that a specific MAP3K, MEKK3, can phosphorylate and activate PKK (phosphorylation depicted in Figure 1 below). MEKK3 has been functionally linked to EGF signaling which may therefore place PKK in a pathway prominently associated with the transformation process in breast cancer. Although PKK expreession cannot readily be detecetd in the majority of human tumor cell lines, its expressed at very high levels in certain human breast tumors (see Figure 2). This, coupled with its limited expression in early murine development indirectly suggests that PKK may be a candidate tumor antigen. We have been in touch with Dr. Lloyd Old's group at Sloan Kettering since they have recently recloned a PKK fragment using sera from breast cancer patients (M.J. Scanlan, personal communication). PKK is therefore, quite fortuitously, a candidate antigen for breast cancer.

It remains to be proven that peptides from PKK actually can be presented by putative protective HLA class II alleles and that this protein may therefore represent an authentic HLA class II restricted tumor antigen.

KEY RESEARCH ACCOMPLISHMENTS

- Complete molecular analyses of all MHC class II genes in patients with early onset breast cancer and controls has been performed
- Two resistance alleles have been identified in women with breast cancer. These represent potential "resistance" genes for breast cancer.
- A putative breast cancer tumor antigen has been cloned and characterized. This putative antigen is a novel protein kinase and is a potential participant in mammary oncogenesis

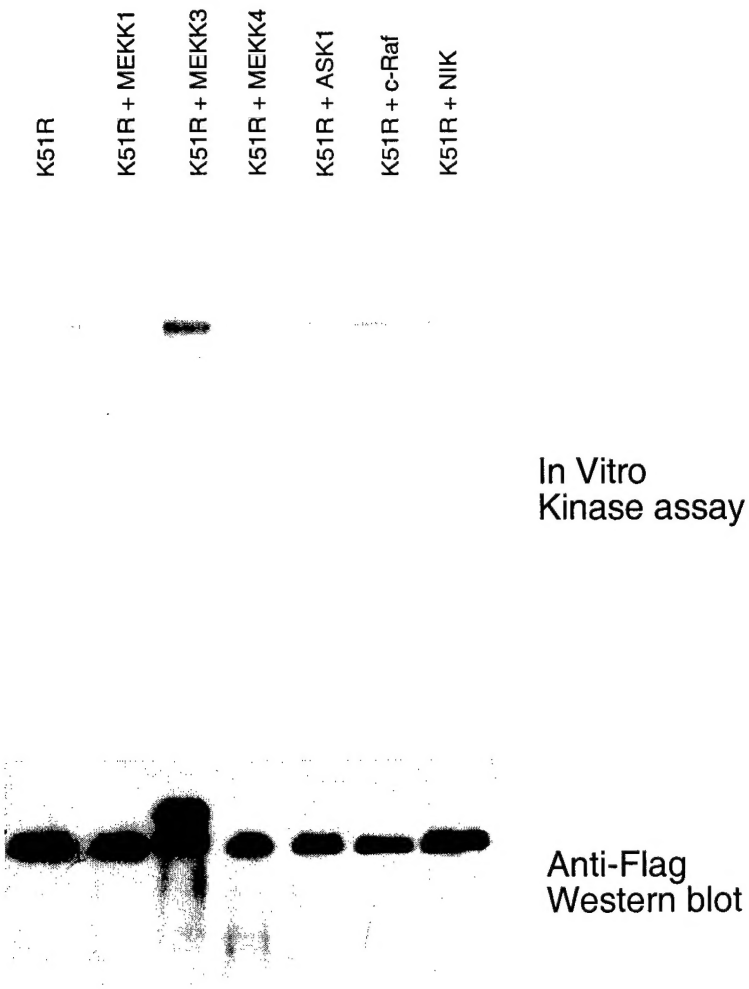
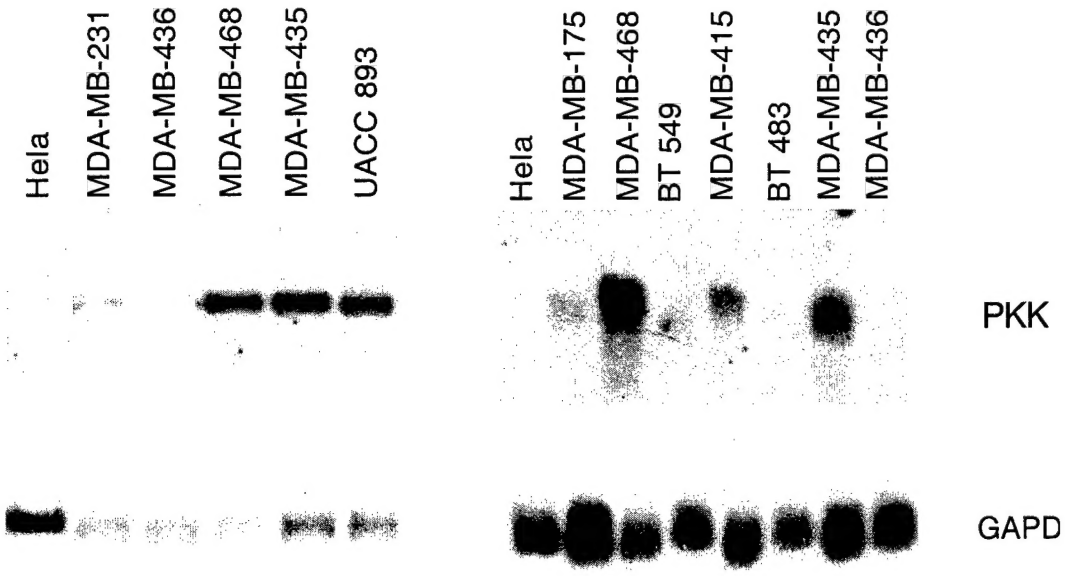


Figure 1. Kinase dead K51R PKK is specifically phosphorylated by MEKK3. In the upper panel phosphorylation is observed in the course of an In vitro kinase assay. In the lower panel, MEKK3 specific phosphorylation can be seen in the Western blot.

Figure 2. Northern blot analysis reveals that PKK is expressed at very high levels in selected human breast cancer cell lines such as MDA-MB 468, MDA-MB-435 and UACC893



REPORTABLE OUTCOMES

1. Chaudhuri S, Cariappa A, Tang M, Bell D. Haber DA, Isselbacher KJ, Finkelstein D, Forcione D, Pillai S.. Genetic susceptibility to breast cancer: HLA DQB*03032 and HLA DRB1*11 may represent protective alleles. Proc. Natl. Acad. Sci. USA. 2000. 97, 11451-11454.

2. PROTECTIVE HLA CLASS II ALLELES IN THE PROGNOSIS AND THERAPY OF HUMAN CANCER. Pillai S, Cariappa A, Forcione D., Chaudhuri S. (Patent applied for).

3. PKK IS A TARGET IN HUMAN CANCER. Pillai S, Chen L, Haider K, Cariappa A, Tang M. (Patent applied for).

4. Support requested in Fall 2001 and obtained for one year from the Avon Foundation for "Genetic Studies on Breast Cancer"

5. Support applied for from the National Institutes of Health for "Studies on Genetic Resistance to Breast Cancer".

CONCLUSIONS

We conclude that there is an extremely significant immunogenetic component to breast cancer. Immune surveillance may be of critical importance in the genesis of breast cancer in young women. There are obvious and very important therapeutic implications which will be considered more fully if we are able to verify and fully establish our current findings. We have also identified a putative breast cancer antigen.

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Genetic susceptibility to breast cancer: HLA DQB*03032 and HLA DRB1*11 may represent protective alleles

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Contributed by Kurt J. Isselbacher, August 2, 2000

Tumors are believed to emerge only when immune surveillance fails. We wished to ascertain whether the failure to inherit putative protective alleles of HLA class II genes is linked to the development of breast cancer. We molecularly typed HLA DPB1, DQB1, DRB1, and DRB3 alleles in 176 Caucasian women diagnosed with early-onset breast cancer and in 215 ethnically matched controls. HLA DQB*03032 was identified in 7% of controls but in no patients with early-onset breast cancer ($P = 0.0001$). HLA DRB1*11 alleles were also significantly overrepresented ($P < 0.0001$) in controls (16.3%) as compared with patients with early-onset breast cancer (3.5%). HLA DQB*03032 and HLA DRB1*11 alleles may have a protective role in human breast cancer.

Although it is likely that genetic susceptibility plays a role in the development of most human cancers, evidence supporting this view hitherto has been obtained in only a small fraction of patients who typically carry germ-line mutations in tumor suppressor genes. In addition to the widely recognized role of acquired alterations in oncogenes and tumor suppressor genes, considerable evidence exists to suggest that the immune system might play a protective role in tumorigenesis. Although immune surveillance is believed to be involved in the elimination of tumors (1, 2), immunotherapeutic approaches to human cancer by and large have proved unsuccessful.

T cell responses are dependent on the inheritance of specific alleles of the highly polymorphic HLA class I and class II genes. Although weak associations of specific HLA alleles with tumors of viral origin have been described (3–10), the relevance of MHC polymorphisms to the broader category of spontaneous nonviral human tumors remains to be established. Somatic alterations in many tumors can contribute to the down-regulation of HLA class I gene expression in tumor cells (11). These alterations potentially could contribute to immune evasion and might represent a discrete event in the multistep paradigm of tumorigenesis. Although HLA class I genes are expressed in all cells, immune responses also require the presentation of antigenic peptides to T cells by HLA class II molecules. These heterodimers primarily are expressed by professional antigen-presenting cells such as macrophages, dendritic cells, and B lymphocytes. Somatic alterations in tumor cells cannot influence the expression of HLA class II genes in dendritic cells and other professional antigen-presenting cells. If, indeed, immune surveillance is important during tumorigenesis, certain individuals who inherit specific alleles of the highly polymorphic HLA class II DPB, DQB, or DRB genes might be resistant to specific types of cancers.

We reasoned that a detailed molecular analysis of HLA DPB, DQB, and DRB alleles in patients with breast cancer and ethnically matched controls might provide information on the potential existence of alleles that could confer susceptibility or resistance to this human cancer. Women with early-onset breast cancer (diagnosed at or before the age of 40) constitute a subset of the population at increased risk for genetic predisposition (12) and, hence, were chosen to test this hypothesis. Such an approach may have the potential to contribute genetically derived insights regarding the role of immune surveillance in cancer.

Methods

Patients. One hundred and eighty-six consecutive women with breast cancer diagnosed before the age of 40 at hospitals in Boston were included in the study (13). Information regarding ethnicity was obtained from each patient. Results from one hundred and seventy-six Caucasian patients are included in this report.

Controls. Two hundred and fifteen healthy Caucasians were included as controls. Ninety-three of these controls have been described earlier (14). The remainder included healthy volunteer blood donors at Massachusetts General Hospital. Information on ethnicity also was obtained from controls.

HLA Class II Typing. Genomic DNA was obtained from lymphoblastoid cell lines derived individually for each subject or from peripheral blood. Genotyping of DRB, DPB1, and DQB1 alleles was performed by using a PCR-sequence-specific oligonucleotide (SSO) technique according to the protocols described in the 11th and 12th International HLA Workshops (15, 16). Details of the PCR and DNA hybridization conditions have been published earlier (14, 17). Briefly, PCRs were performed in a total volume of 200 μl and included 100 ng of genomic DNA and a reaction mixture [200 pM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 2 mM $MgCl_2$, 10 mM Tris·HCl (pH 8.0), 50 mM KCl, 0.001% (wt/vol) gelatin, and 2.5 units of *Taq* DNA polymerase (Fisher)]. Samples were denatured at 96°C for 6 min followed by 40 cycles of amplification. The annealing temperature for the generic DRB1 alleles was 55°C and 60°C, respectively, for DRB1 and DRB3 group-specific amplifications, 60°C for DP, and 55°C for DQ. After confirmation of the PCR products on a 1.5% agarose gel, up to 5 μl of the product was spotted on nylon membrane filters. The filters were prehybridized overnight at 54°C in a buffer containing 3 M tetramethylammonium chloride, 50 mM Tris·HCl (pH 8.0), and 2 mM EDTA/5× Denhardt's solution/0.1% SDS/100 μg/ml of salmon sperm DNA. SSO probes were 5′ end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Hybridization was carried out at 54°C for 2 h. The filters were washed twice at room temperature for 15 min each in a solution containing 2× SSPE [standard saline

Abbreviation: SSO, sequence-specific oligonucleotide.

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Table 1. DPB1 alleles in controls and breast cancer

Allele	Controls (n = 207)	Breast cancer (n = 157)	P value†
DPB1*0101	8.2% (17)	5.1% (8)	0.2981
DPB1*0201	16.4% (34)	22.3% (35)	0.1776
DPB1*0202	1.4% (3)	0.6% (1)	0.6371
DPB1*0301	17.4% (36)	17.2% (27)	1.0000
DPB1*0401	52.2% (108)	36.9% (58)	0.0042
DPB1*0402	25.6% (53)	21.0% (33)	0.3218
DPB1*0501	1.9% (4)	4.5% (7)	0.2184
DPB1*0601	1.0% (2)	4.5% (7)	0.0429
DPB1*0801	1.9% (4)	1.9% (3)	1.0000
DPB1*0901	0.0% (0)	1.3% (2)	0.1854
DPB1*1001	6.3% (13)	1.3% (2)	0.0172
DPB1*1101	1.9% (4)	3.2% (5)	0.5077
DPB1*1301	4.3% (9)	0.6% (1)	0.0479
DPB1*1401	3.4% (7)	2.5% (4)	0.7632
DPB1*1501	1.4% (3)	2.5% (4)	0.4704
DPB1*1601	1.9% (4)	1.3% (2)	0.702??
DPB1*1701	1.9% (4)	3.8% (6)	0.3388
DPB1*1801	0.5% (1)	2.5% (4)	0.1700
DPB1*1901	0.5% (1)	0.6% (1)	1.0000
DPB1*2001	4.8% (10)	5.7% (9)	0.8130
DPB1*2201	0.0% (0)	0.6% (1)	0.4313
DPB1*2301	23.7% (49)	19.1% (30)	0.3077
DPB1*2401	1.9% (4)	1.9% (3)	1.0000
DPB1*2501	1.4% (3)	5.7% (9)	0.0350
DPB1*2601	1.0% (2)	3.2% (5)	0.1459
DPB1*2701	2.9% (6)	1.9% (3)	0.7374
DPB1*2801	0.0% (0)	0.6% (1)	0.4313
DPB1*2901	1.4% (3)	5.1% (8)	0.0620
DPB1*3101	1.9% (4)	0.0% (0)	0.1371
DPB1*3201	1.9% (4)	7.0% (11)	0.0297
DPB1*3301	0.5% (1)	5.1% (8)	0.0061
DPB1*3401	0.0% (0)	0.6% (1)	0.4313
DPB1*3501	1.4% (3)	1.3% (2)	1.0000

†Uncorrected two-tailed P value (Fisher's Exact Test).

phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)] and 0.1% SDS followed by three washes for 10 min at 58°C in a buffer containing 3 M tetramethylammonium chloride, 50 mM Tris·HCl, 2 mM EDTA, and 0.1% SDS. Each filter was exposed twice for autoradiography, once for 1–2 h, and subsequently for 14–16 h. Reactivity was graded visually, using a scale recommended by the 11th International HLA Workshop (15). Controls included previously typed samples.

Generic typing of DRB1 and DRB3 loci was performed by using a single pair of generic primers for PCR amplification followed by hybridization of filters with 29 different group-identifying SSOs. For group-specific DRB typing, genomic DNA was amplified with five different 5′ primers specific for DRB1-DR1, DRB1-DR2, DRB1-DR4, the DRB1-DR52-associated group and the DRB3-DR52 group. The 3′ primer in the above cases was the same as that used for generic DRB typing. A total of 50 different SSOs were used in the group-specific typing studies. Five SSOs were used for DRB1-DR1 (DRB1*0101–03), 12 were used for DRB1-DR2 (DRB1*1501–03, DRB1*1601–02), 9 were used for DRB1-DR4 (DRB1*0401–0411), 12 were used for the DRB1-DR52-associated group (DRB1*0301/02, DRB1*0801–0804, DRB1*1101–04, DRB1*1201/02, DRB1*1301–05, DRB1*1401–08), and 12 were used for the DRB3-DR52 group (DRB3*0101, DRB3*0201/0202 and DRB3*0301).

Generic primers were used for PCR amplification of DPB1 and DQB1 loci. Twenty-five SSOs were used to type 36 DPB1 alleles, and 20 SSOs were used for 17 DQB1 alleles. The use of multiple oligonucleotide probes facilitated definitive identification of negative and positive alleles.

Validation. Selected PCR products were subcloned (five subclones were picked after transformation with DNA from each ligation mixture), and individual clones were sequenced to confirm the validity of allele assignment by the PCR/SSO typing approach.

Statistical Analysis. Two-tailed uncorrected P values were reported by using Fisher's exact test for the analyses of HLA class II allele frequencies. The relative risk was calculated as an odds ratio by using the approximation of Woolf (18). P values were corrected for the number of comparisons essentially by using a modified Bonferroni correction as suggested by Svejgaard and Ryder (19). The number of alleles assayed from a specific PCR amplification reaction was used as the basis for the number of comparisons made. Alleles for which the combined frequency in patients and controls was less than 1 were not included in the number of comparisons. In the case of the DPB1 locus, the number of comparisons made was 33. Thus, the nominal level for comparison was $P = 0.0015$. In the case of the DQB1 locus, the number of comparisons made was 16 and the nominal level for comparison was $P = 0.0031$. In the case of the DRB1 locus, the number of comparisons was 31 and the nominal level for comparison was $P = 0.0016$. In the case of the DRB3 locus, the number of comparisons was 3 and the nominal level for comparison was $P = 0.0166$.

Results

We noted two strong, negative associations of HLA class II alleles with early-onset breast cancer, one with a DQB1 allele and another with a set of DRB1 alleles. A weaker, positive association was noted with a single DRB3 allele.

No strong negative or positive associations were noted for any DPB1 alleles in patients with breast cancer (Table 1). A weak negative association was seen for DPB1*0401 ($P = 0.0042$; corrected $P = 0.1386$, not significant), and a weak positive association was noted for DPB1*3301 ($P = 0.0061$; corrected $P = 0.2013$, not significant).

However, we found that 14 of 199 controls but none of 176 patients with breast cancer inherited the DQB*03032 allele ($P = 0.0001$) (Table 2). The relative risk was 0.0358. The corrected P

Table 2. DQB1 alleles in controls and breast cancer

Allele	Controls (n = 199)	Breast cancer (n = 176)	P value†
DQB1*0201	30.7% (61)	39.8% (70)	0.0663
DQB1*0301	40.2% (80)	36.4% (64)	0.4584
DQB1*0302	28.1% (56)	20.5% (36)	0.0930
DQB1*03032	7.0% (14)	0.0% (0)	0.0001‡
DQB1*0305	1.0% (2)	0.6% (1)	1.0000
DQB1*0401	0.5% (1)	3.4% (6)	0.0545
DQB1*0402	4.5% (9)	1.1% (2)	0.0670
DQB1*0501	17.1% (34)	21.6% (38)	0.2946
DQB1*0502	3.5% (7)	4.0% (7)	1.0000
DQB1*05031	6.5% (13)	3.4% (6)	0.2381
DQB1*05032	0.5% (1)	0.0% (0)	1.0000
DQB1*0504	0.0% (0)	0.0% (0)	—
DQB1*0601	2.0% (4)	5.7% (10)	0.0986
DQB1*0602	26.6% (53)	22.7% (40)	0.4035
DQB1*0603	10.0% (20)	10.2% (18)	1.0000
DQB1*0604	4.5% (9)	3.4% (6)	0.6105
DQB1*0605	1.0% (2)	4.5% (8)	0.0506

†Uncorrected two-tailed P value (Fisher's Exact Test).
‡Significant after correction; nominal value for comparison, $P \leq 0.0031$.

Table 3. DRB1 alleles in controls and breast cancer

Allele		Controls (n = 215)	Breast cancer (n = 173)	P value†
DR1	*0101	11.6% (25)	13.3% (23)	0.6442
	*0102	4.2% (9)	5.8% (10)	0.4875
	*0103	2.8% (6)	5.8% (10)	0.1984
DR2	*1501	27.9% (60)	20.2% (35)	0.0963
	*1502	0.9% (2)	1.7% (3)	0.6597
	*1503	0.9% (2)	0.6% (1)	1.0000
	*1601	2.8% (6)	2.9% (5)	1.0000
	*1602	1.4% (3)	1.2% (2)	1.0000
DR3	*0301–02	20.5% (44)	30.1% (52)	0.0333
DR4	*0401	6.5% (14)	4.0% (7)	0.3684
	*0402	5.6% (12)	4.6% (8)	0.8183
	*0403	1.4% (3)	0.0% (0)	0.2569
	*0404	10.7% (23)	11.6% (20)	0.8711
	*0405	1.9% (4)	0.6% (1)	0.3865
	*0407	2.3% (5)	1.2% (2)	0.4682
	*0408	7.4% (16)	6.4% (11)	0.8413
	*0409	0.0% (0)	0.0% (0)	—
	*0410	0.0% (0)	0.0% (0)	—
	*0411	0.0% (0)	0.0% (0)	—
DR7	*0701	19.5% (42)	24.9% (43)	0.2189
DR8	*0801–04	4.2% (9)	6.4% (11)	0.3632
DR9	*0901A/B	2.8% (6)	0.6% (1)	0.1370
DR10	*1001	0.9% (2)	1.2% (2)	1.0000
DR11	*1101–04	16.3% (35)	3.5% (6)	<0.0001‡
DR12	*1201–02	3.2% (7)	5.2% (9)	0.4425
DR13	*1301	5.1% (11)	0.6% (1)	0.0147
	*1302	10.2% (22)	12.1% (21)	0.6263
	*1303	0.9% (2)	4.6% (8)	0.0268
	*1304	0.0% (0)	0.0% (0)	—
	*1305	0.0% (0)	0.6% (1)	0.4459
DR14	*1401	5.1% (11)	1.2% (2)	0.0441
	*1402	0.0% (0)	0.6% (1)	0.4459
	*1403	6.5% (14)	7.5% (13)	0.6944
	*1404	1.9% (4)	1.2% (2)	0.6961
	*1405	2.8% (6)	5.8% (10)	0.1984
	*1406	0.0% (0)	0.0% (0)	—
	*1407	0.0% (0)	0.0% (0)	—
	*1408	0.0% (0)	0.0% (0)	—

†Uncorrected two-tailed P value (Fisher's Exact Test).
‡Significant after correction; nominal value for comparison, $P \leq 0.0016$.

value is 0.0016, which remains highly significant. No other DQB alleles were significantly over- or underrepresented in the control group.

The only allele or group of alleles at the DRB1 locus that was significantly different between patients and controls was DRB*11, which was found in 35 controls but in only 6 patients with breast cancer ($P < 0.0001$) (Table 3).The relative risk was 0.1846. The corrected P value for this negative association is < 0.0030, which is also highly significant. At least 34 different DRB1*11 alleles have been described in recent years, some of which remain to be confirmed (20). The frequencies with which many of these newer alleles are seen in Caucasians has not been established, and it is likely that many of them will be extremely rare. This group of alleles deserves extremely thorough scrutiny in future studies.

DRB1*11 alleles are not in linkage disequilibrium with DQB*03032. In Caucasians, DQB*03032 is in very weak linkage disequilibrium with DRB1*0701, DRB1*0901, and DRB1*1602. It is clear from Table 3 that the negative association with DQB*03032 does not represent linkage disequilibrium with a known DRB1 gene.

More than half the patients with breast cancer (94 of a total of 171, 55%) and a substantial but smaller proportion of the

Table 4. DRB3 alleles in controls and breast cancer

Allele	Controls (n = 208)	Breast cancer (n = 171)	P value†
DRB3			
*0101	24.5% (51)	25.1% (43)	0.9053
*0201/*0202	40.9% (85)	55.0% (94)	0.0072‡
*0301	7.7% (16)	9.4% (16)	0.5823

†Uncorrected two-tailed P value (Fisher's Exact Test).
‡Significant after correction; nominal value for comparison, $P \leq 0.0166$.

controls (85 of 208, 40.9%) inherited DRB3*02 (Table 4). The P value for this positive association was 0.0072. The corrected P value was 0.0216, which remains significant.

One concern in any population-based study is the role of ethnicity. The vast majority of the patients and controls originally collected for study were Caucasians. A few individuals in each of these groups were of Asian, Hispanic, or African American origin and were excluded from the study. The patient group contained 17 Jewish subjects, and 13 Jewish controls were studied. When the data were analyzed separately excluding Jewish patients and controls, the negative associations of DQB*03032 and the DRB1*11 in breast cancer remained highly significant (DQB*03032, $P = 0.0002$; DRB1*11, $P = 0.0006$). The numbers of Jewish patients and controls were insufficient for this subgroup to be analyzed separately in a statistically meaningful manner. None of the Jewish patients or controls inherited DQB*03032. It was nonetheless intriguing that 5 of 13 Jewish controls (38.5%) and 0 of 17 Jewish patients (0%) inherited DRB1*11 ($P = 0.0090$).

Discussion

It has long been recognized that genetic susceptibility to cancer in part may be due to inherited variations in MHC genes. Inheritance of specific MHC class II genes may promote the generation of specific T cell help for the elimination of pathogens and, thus, may be correlated with resistance to tumors, particularly those linked to viral etiologies. An example of such an association is observed in the regression of cottontail rabbit papilloma virus-induced warts in rabbits that inherit a particular MHC class II DQ α restriction fragment length polymorphism (21).

We wanted to perform a study with sufficient power (in terms of numbers of subjects and the range of alleles examined) to ascertain whether protective HLA class II alleles could be identified in the context of a human cancer of presumed nonviral origin. Such alleles, if they exist, theoretically would fit the definition of being dominant tumor suppressor genes (10). No studies on HLA class II alleles in breast cancer have been reported to date. We chose to study early-onset breast cancer based on the presumption that genetic susceptibility would be revealed more easily in this subset of patients. Our control group included individuals of both sexes. The most power perhaps would have been achieved by selecting a cohort of ethnically matched elderly women with no history of cancer.

Our studies suggest that DQB* 03032 and DRB1*11 alleles may represent resistance alleles for early-onset breast cancer. It is theoretically possible that DQB*03032 is in linkage disequilibrium with an unidentified growth-regulating gene, a polymorphic allele of which dominantly suppresses mammary tumorigenesis. If such a polymorphic-linked tumor suppressor gene exists, an allele of this gene would be expected to form an extended haplotype in conjunction with DQB*03032 and a specific linked DRB1 allele. The failure to note a negative association in breast cancer with any one of the three DRB1 alleles known to be in linkage disequilibrium with DQB*03032 in Caucasians argues against the theoretical possibility that DQB*03032 is in linkage disequilibrium with an unknown dominant tumor suppressor gene. The possible existence

of such a linked dominant tumor suppressor, however, has not been ruled out.

Although we have examined a relatively large cohort of women who developed breast cancer at or before the age of 40, clearly more extensive studies need to be conducted. It is possible that our results are meaningful only for early-onset breast cancer, and it remains to be seen whether similar negative associations will be revealed in studies on a more broadly selected group of patients. The negative association noted for DRB1*11 even in the very small subgroup of Jewish subjects is intriguing, and, clearly, a large study needs to be undertaken involving Jewish women with breast cancer and ethnically matched controls. Although, at the very outset, we suspected that negative associations of HLA class II genes might be observed in early-onset breast cancer, we had no *a priori* reason to focus on any specific allele. Our study, therefore, should be considered exploratory and requires to be confirmed by a study on a distinct set of patients and controls.

If, indeed, the negative associations described here are supported in subsequent independent studies, it would strengthen the view that yet to be identified protective mammary tumor-specific peptides lodge in the antigen-binding grooves of specific HLA class II heterodimers in resistant individuals. Peptides bound to DQB*03032 and DRB1*11 may be presented to T cells in resistant individuals. Typing of these HLA class II alleles may prove of prognostic value. The introduction of these specific alleles into hematopoietic stem cells or into dendritic cells in breast cancer patients eventually might be considered if their protective importance is confirmed.

A significant positive association was noted with DRB3*02 alleles, although these alleles were also frequently inherited by controls. The positive association of specific HLA class II alleles in any form of cancer may reflect the role of specific HLA class II molecules either in promoting chronic inflammation or in influencing the development of a hole in the T cell repertoire during thymic education. Although lymphocytic infiltration and fibrosis are seen frequently in human breast cancer, there is little clinical evidence to suggest that breast cancer in women develops in a setting of chronic inflammation. In any individual, $CD4^+CD8^+$ double-positive thymocytes bearing T cell receptors capable of avidly recognizing self-MHC molecules are eliminated. This deletional process is important in maintaining self-tolerance but can be a two-edged sword. The presentation of self-peptides by specific breast cancer-associated HLA class II alleles may eliminate certain T cell clones that might have the potential to respond to specific tumor antigens.

The role of endocrine and genetic factors in the pathogenesis of breast cancer is widely appreciated. In recent years, considerable molecular knowledge has accrued on the genetic susceptibility to breast cancer. In a small subset of patients, germ-line mutations in tumor suppressor genes have been demonstrated. These genes include p53 (22, 23), BRCA1 (13, 24–26), and BRCA 2 (26); other susceptibility loci that remain to be characterized may also play a role.

Immune surveillance potentially could be directed against mutant self-proteins (27) or against proteins expressed in a highly tissue-specific manner in the tissue of origin of the tumor (28, 29). A third category of tumor antigen is represented by proteins that are poorly expressed during development, but are expressed at high levels in some tumors (30, 31), including breast carcinomas (32). Although immune surveillance might play a role in eliminating incipient breast malignancies, no direct evidence exists to support such a postulate. The confirmation of the existence of protective HLA class II alleles in any form of human cancer, including breast cancer, would lend genetic support to the concept of immune surveillance as a critical component involved in tumorigenesis.

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